Heme-Solvent Coupling: A Mössbauer Study of Myoglobin in Sucrose

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ABSTRACT The Mössbauer effect of ^{57}Fe-enriched samples was used to investigate the coupling of 80% sucrose/water, a protein-stabilizing solvent, to vibrational and diffusive modes of the heme iron of CO-myoglobin. For comparison we also determined the Mössbauer spectra of $K_4{}^{57}Fe(CN)_6$ (potassium ferrocyanide, PFC), where the iron is fully exposed in the same solvent. The temperature dependence of the Mössbauer parameters derived for the two samples proved to be remarkably similar, indicative of a strong coupling of the main heme displacements to the viscoelastic relaxation of the solvent. We show that CO escape out of the heme pocket couples to the same type of fluctuations, whereas intramolecular bond formation involves solvent-decoupled heme deformation modes that are less prominent in the Mössbauer spectrum. With respect to other solvents, however, sucrose shows a reduced viscosity effect on heme displacements and the kinetics of ligand binding due to preferential hydration of the protein. This result confirms thermodynamic predictions of the stabilizing action of sucrose by a dynamic method.

INTRODUCTION

A unique apolar environment is one of the essential aspects in the catalytic enhancement of biochemical reactions. On the other hand, the binding site has to be accessible from the outside. The ligands enter and leave the protein structure, which requires a pathway possibly gated by structural fluctuations that couple the active site to the protein surface. The heme group of myoglobin and of most other heme proteins is buried in a cleft of the apo-structure.

In the case of myoglobin this serves to prevent the oxidation of the ferrous iron, which is catalyzed by water. The shielding from the solvent is, however, not perfect as the heme-propionate side chains protrude into the solvent. It is important to assess whether these contacts affect the intramolecular mobility of the heme and, in turn, the kinetics of ligand binding. Structural interpretations predict that the salt bridge formed by Arg45 and the heme-6-propionate side chain controls the access to the heme pocket (Ringe et al., 1984; Perutz, 1989).

The Mössbauer effect, using ^{57}Fe-enriched samples, allows one to probe the dynamics of the heme iron. The so-called recoilless nuclear resonant absorption of γ-rays has so far led to wide application in the research of protein dynamics, using radioisotopes as Mössbauer γ-ray sources, such as ^{57}Co. The Mössbauer effect in proteins has the two characteristic features of a rapid decrease of the Debye-Waller factor above a characteristic temperature ($\sim$200 K) and the appearance of quasielastic line-broadening in the energy range of a few neV (Parak and Formanek, 1971; Keller and Debrunner, 1980; Parak et al., 1981, 1982; Nowik et al., 1983). These results were interpreted in terms of protein-specific structural jumps and diffusion between conformational substates (Knapp et al. 1982; Nadler and Schulten, 1984). It is thus of interest to establish whether the characteristic temperature of 200 K is a property of the protein alone or whether it reflects mainly the heme-solvent coupling. To clarify this point we have performed a Mössbauer study of myoglobin in a viscous solvent, 80% sucrose/water, which has a glass temperature well above 200 K.

Sucrose belongs to the small set of compounds that were selected by organisms, ranging from single cells to amphibians and higher plants, to resist dehydration, osmotic shock, and freezing at subzero temperatures (Yancey et al., 1982; Carpenter and Crowe, 1988a,b; Crowe et al., 1996). The carbohydrate is also well known as a potent stabilizer of proteins in their native state. These features have been attributed to the ability of sucrose and other carbohydrates to replace or complement the hydration shell of proteins (Carpenter and Crowe, 1989). The thermodynamic analysis of Timasheff and collaborators (Timasheff, 1993; Lin and Timasheff, 1996) suggests instead that sucrose enhances the tension at the protein-solvent interface and is thus excluded preferentially from the protein domain.

The suggested partial demixing of the solvent should be readily observable in those dynamical parameters that are affected by the microviscosity at the protein surface. In a previous study we have characterized the kinetics of ligand binding to myoglobin in various solvents, including 80% sucrose/water (Kleinert et al., 1998). Below we show that the kinetics of ligand binding to myoglobin and Mössbauer spectroscopy of the heme binding site show parallel effects in response to changes in the co-solvent composition and solvent viscosity. The behavior of myoglobin and hemoglobin in viscous solvents and glasses has been investigated previously using other spectroscopic techniques (Beece et

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al., 1980; Ansari et al., 1992; Hagen et al., 1995, 1996; Gottfried et al., 1996).

MATERIALS AND METHODS

Sample preparation

To investigate the dynamics of the 80% sucrose/water solvent by Mössbauer spectroscopy, $K_4{}^{57}Fe(CN)_6$ (potassium ferrocyanide, abbreviated PFC) was added to the solvent. PFC and sucrose were dissolved separately in an excess of water. The sucrose/water mixture was stirred at 70°C until it was homogeneous and clear. The PFC solution was added and water was evaporated from the mixture to obtain a concentration of 3.3% PFC and 96.7% sucrose/water solution (concentration of sucrose/water, 78.7:21.3, w/w). The sucrose concentration was kept slightly below 80%, because the risk of crystallization at ~0°C or below rises dramatically at 80%. The absorption corrections due to different ^{57}Fe concentrations were investigated using three different samples: the first one containing ^{57}Fe-enriched potassium-ferrocyanide, $n_{57Fe} = 21.40 \times 10^{17}$ cm^{-2}, a second one with natural PFC, $n_{57Fe} = 5.96 \times 10^{17}$ cm^{-2}, and the third one with exactly the same iron concentration as used for the myoglobin sample, $n_{57Fe} = 3.24 \times 10^{17}$ cm^{-2}, where n_{57Fe} is the number of ^{57}Fe atoms per cm^2 seen by the γ-rays emitted from the source. The respective data sets were separately analyzed and averaged afterwards as no systematic differences were found.

To obtain a sample with MbCO in sucrose solution (MbCO/SUC), 20 mg of ^{57}Fe-enriched horse myoglobin in 50 mM phosphate buffer at pH 7 was reduced by sodium dithionite under N_2 atmosphere. CO-ligated myoglobin was obtained by exchanging the N_2 gas with CO. The MbCO solution was concentrated to ~100 ml. Then, 160 mg of sucrose was dissolved in 300 ml of H_2O at 80°C under CO atmosphere and concentrated to 200 ml. The MbCO and sucrose solutions were mixed in the sample holder. This solution was kept over silica gel at 43°C and dried to a final concentration of 12.5% CO-ligated horse myoglobin and 87.5% sucrose/water solution (w/w). The sealed sample was cooled in an ice bath. Then the temperature was slowly decreased to −18°C. Finally, the sample was stored in liquid nitrogen.

Data acquisition and analysis

Mössbauer spectra were measured with a $^{57}CoRh$ source emitting 14.4 keV of γ-radiation. Some of the quanta were absorbed resonantly by the ^{57}Fe atoms in the sample. The extent of resonant absorption in the sample was measured in transmission geometry. Energy-resolved measurements were achieved by moving the emitter relative to the absorber with variable velocity v, using a Doppler drive. During the measurement, the velocity was varied in the range of either ±3 or ±12 mm/s. The absorption spectra of the PFC samples and the MbCO/SUC sample were measured in a cryostat with a temperature stability of ±0.1 K. Several series were performed all starting at 100 K and continuing to temperatures up to 265 K. The temperature was raised by 1 K/min and kept constant at each for several minutes before starting the measurement.

We measured the transmission $D(v)$ of the sample, which should, in the simplest case, exhibit a Lorentzian shape, the Fourier transform of an exponential decay process, characterized by a width Γ_v and a central position on the velocity axis v_0 (Greenwood and Gibb, 1971):

$$D(v) = 100 \cdot \left(1 - \frac{I(v_{max}) - I(v_0)}{I(v_{max})} \cdot \frac{\left(\frac{\Gamma_v}{2}\right)^2}{\left(\frac{\Gamma_v}{2}\right)^2 + (v - v_0)^2} \right) \quad (1)$$

$D(v)$ is the transmission of Mössbauer radiation in percent, v is the velocity in mm/s of the source of the γ-radiation relative to the sample. $I(v_0)$ denotes the maximal number of counts at v_0. $I(v_{max})$ is the number of counts at maximal velocity. The Doppler velocity can be converted to a conventional energy (frequency) scale by $E = h\nu = (v/c) \times 14.4$ keV. Thus, a velocity of $v = 0.1$ mm/s corresponds to an energy of 4.8 neV. The minimal width of the Lorentzian is given by the natural linewidth, $\Gamma_N = h/(2\pi\tau_N) \approx 0.1$ mm/s, where and $\tau_N = 141$ ns denotes the lifetime of the excited state of the ^{57}Fe nucleus. As both absorber and emitter contribute their natural linewidth, the effective instrumental width in transmission geometry is $2\Gamma_N = 0.2$ mm/s. Chemical inhomogeneities of the source and/or the sample and dynamical processes on a time scale comparable to τ_N lead to a further broadening of the resonance line Γ_v.

Another important quantity is the velocity integrated transmission, the total area covered by the resonance line, which for a Lorentzian is given by:

$$A = \pi I(v_0) \frac{\Gamma_v}{2} = f_s f_a n_{57Fe} \sigma, \quad (2)$$

where f_s denotes the fraction of γ-quanta emitted from the source without recoil (Lamb-Mössbauer factor of the source), f_a is the fraction of γ-quanta absorbed by the sample without recoil (Lamb-Mössbauer factor of the absorber which is the sample), n_{57Fe} is the number of ^{57}Fe atoms per cm^2, and σ is the absorption cross section of a single ^{57}Fe atom. Most conclusions derived from Mössbauer experiments are based on the Lamb-Mössbauer factor f_a, which, in the Gaussian approximation, can be written as:

$$f_a = \exp(-k^2\langle x^2 \rangle), \quad (3)$$

where $k = 2\pi/\lambda = 7.3\ A^{-1}$ is the wave vector of the 14.4 keV γ-radiation emitted by the Mössbauer source, and $\langle x^2 \rangle$ denotes the mean square displacement of the iron atoms in the sample. f_a equals unity if the absorber nucleus does not move relative to the emitter. The displacements thus refer exclusively to dynamical disorder; static disorder is not detected. The $\langle x^2 \rangle$ values were determined from:

$$\ln A = -k^2\langle x^2 \rangle + \ln(f_s n_{57Fe} \sigma). \quad (4)$$

To get absolute values for $\langle x^2 \rangle$, it is assumed that the mean square displacements decrease linearly with temperature below 180 K and extrapolate to the classical value of $\langle x^2 \rangle = 0$ at $T = 0$. This way the second term of Eq. 4 is eliminated. Assuming that the heme iron is coupled to a system of harmonic oscillators with mass m, frequencies ν_i and coupling coefficients β_i, one obtains the expression (Housley and Hess, 1966):

$$\langle x^2 \rangle = h/(6\ m)\Sigma(\beta_i^2/\nu_i)\coth(h\nu_i/2\ k_B T) \quad (5)$$

which is proportional to $1/\nu_i^2$ in the high-temperature limit, emphasizing the low-frequency modes.

The observed spectra are not symmetric with respect to $v = 0$. This shift in the center of gravity, the isomer shift $v_0 = \delta S$, has two major contributions, the chemical shift δS_{chem}, which depends on the particular chemical environment of the heme iron, and the so-called second-order Doppler shift δS_{SOD}, which is proportional to $\langle v^2 \rangle$, the mean squared velocity of the heme iron (Reinisch et al., 1985):

$$\delta S_{SOD} = -E_\gamma \frac{\langle v^2 \rangle}{2c^2} \quad (6)$$

where $E_\gamma = 14.4$ keV is the energy of the γ-radiation. In a harmonic coupling model one obtains for the mean squared velocity (Housley and Hess, 1966):

$$\langle v^2 \rangle = 1/(2\ m)\Sigma\beta_i^2 h\nu_i \coth(h\nu_i/2\ k_B T) \quad (7)$$

Note that this expression is biased toward the high-frequency vibrational components in contrast to Eq. 5. The total isomeric shift δS is then given by:

$$\delta S = \delta S_{chem} + \delta S_{SOD} \quad (8)$$

RESULTS

Figs. 1 and 2 show the Mössbauer spectra of the iron salt PFC and of myoglobin in 80% sucrose/water, respectively. In the former, despite the low-spin II+ state of the iron, the electric quadrupole splitting of the energy levels is small because of the octahedral symmetry of the iron environment. The PFC data could thus be fitted using a single Lorentzian line characterized by the width Γ_v. In contrast, the iron atom in MbCO, which is also in a low spin state, resides in a highly asymmetric environment resulting in a significant quadrupole splitting of the absorption line. In this case the data can be fitted by a superposition of two Lorentzians of equal widths, Γ_v, and equal minima, i.e., a Lorentzian doublet. Fig. 3 displays the temperature dependence of the linewidths. Γ_v of the PFC sample remains constant at low temperatures, reflecting the instrumental resolution, but increases above 250 K. The MbCO/SUC doublet has a slightly lower width at low temperatures (less inhomogeneous broadening), and the corresponding increase in Γ_v starts at the slightly lower temperature of 240 K. The line broadening at the onset temperature reflects diffusive motions that become resolved when the corresponding relaxation times have reached the level of $10 \times \tau_N$, a few microseconds.

Two kinds of diffusion are expected to occur: bound diffusion of the heme group within the protein and diffusion of the whole protein molecule (Keller and Debrunner, 1980). The latter seems to be less likely because the protein exhibits a lower onset temperature than the smaller and thus faster PFC molecule. Expanding the velocity window of the spectra shows that a single Lorentzian component is not perfectly adequate to fit the data above 240 K. This effect is most pronounced for the MbCO/SUC sample (Fig. 2, right). Fitting attempts, introducing a second Lorentzian doublet, yield a much broader line of ~2 mm/s. It is also conceivable that the high-velocity wings in the spectra represent non-Lorentzian corrections to the first doublet rather than a well distinguished second component (Parak et al., 1989).

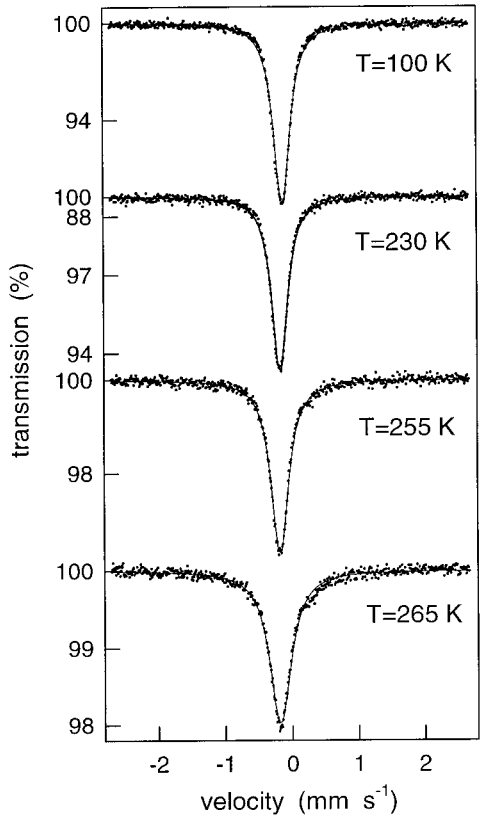

FIGURE 1 Mössbauer spectra of potassium-ferrocyanide (PFC) in 80% sucrose/water at different temperatures. The spectra were measured with a maximal velocity of 3 mm/s, and they were fitted by Lorentzians.

The mean square displacements of the ^{57}Fe nucleus versus the temperature, derived from Eqs. 3 and 4, are depicted in Fig. 4. The figure compares data obtained with deoxymyoglobin crystals (Parak et al., 1982), MbCO/SUC, and PFC. The displacements show a common linear increase at low temperatures but start to diverge above 150 K. For the myoglobin crystals the most pronounced increase takes place at ~200 K. Similar features were observed with aqueous (frozen) myoglobin solutions (Keller and Debrunner, 1980). Comparing these data with our results on MbCO/SUC, we notice a clear up-shift in the main transition to ~240 K. A minor deviation from the harmonic behavior occurs at 210 K. The iron in PFC exhibits larger displacements than MbCO/SUC at low temperatures, but the onset temperatures of anharmonic behavior, 230–240 K, are similar. The up-shift in the transition temperatures may reflect the higher viscosity of the sucrose solution relative to crystal water. The two-component analysis of the spectra, mentioned above, reduces the absolute values of the displacements by ~20% and may up-shift the transition temperature by ~5°.

Fig. 5 illustrates how the center of gravity v_0 of spectra in two solvents changes with the temperature. The figure compares data obtained with myoglobin in 75% glycerol/water (Franke, 1992) with data obtained with myoglobin and PFC in 80% sucrose/water. For optimal superposition and to account for the difference in chemical shift between PFC and myoglobin, a constant of 0.285 mm/s was added to the central shift of PFC. These systems do not exhibit any low-lying electronic states whose population would change with temperature (Trautwein et al., 1970). The chemical shift displayed by these systems is thus assumed to be temperature independent. The differences in the temperature behavior, displayed in Fig. 5, are thus assigned to variations in the vibronic coupling of the heme to its environment. It follows that the composition of the solvent affects the vibrational spectrum of the heme in myoglobin. Furthermore, the solvent-exposed PFC molecule displays nearly the same temperature dependence in the central shift as myoglobin. In fact, the differences in $\delta S(T)$ observed between PFC and myoglobin in the same solvent are smaller than those found with myoglobin in 75% glycerol/water and 80% sucrose/water.

Information about the relevant modes is obtained using the harmonic analysis of Eqs. 7 and 8. We first fit the 75%

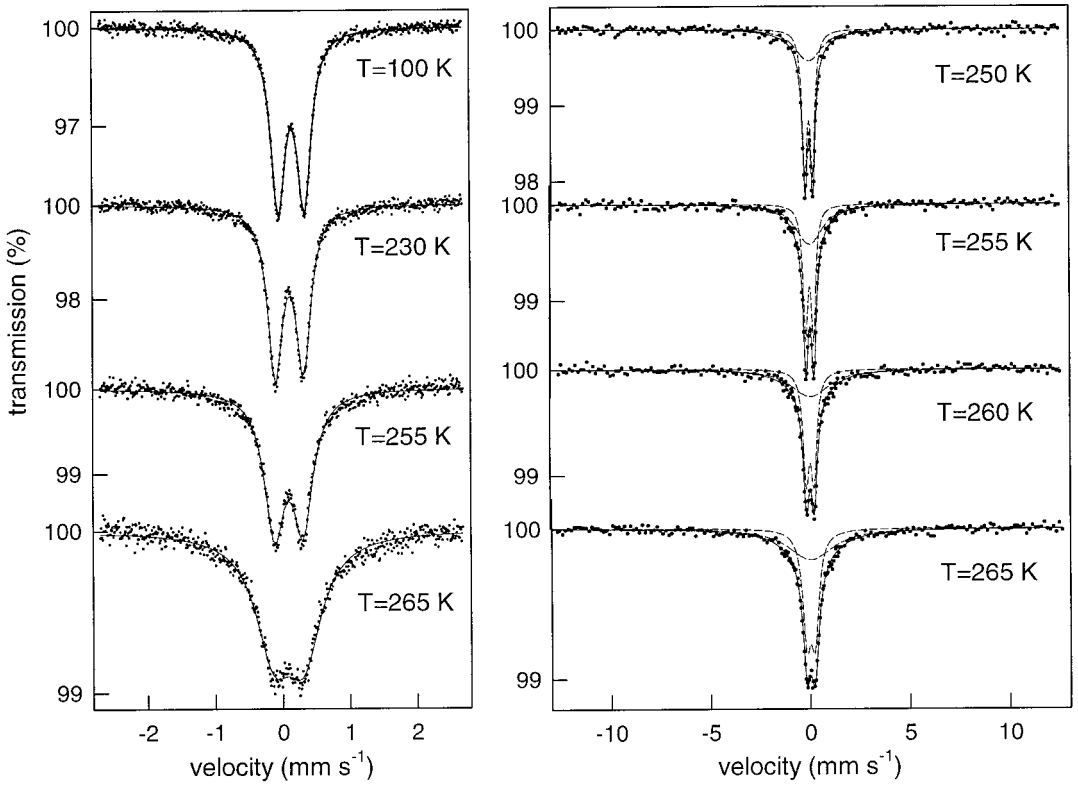

FIGURE 2 MbCO in 80% sucrose/water at different temperatures. The spectra were measured with a maximal velocity of 3 mm/s (*left*) and 12 mm/s (*right*), and they were fitted by narrow and broad Lorentzian doublets.

glycerol/water data, which cover the widest temperature range using a single effective mode. This procedure yields an effective frequency near 440 ± 20 cm^{-1}. However, as Fig. 5 (dashed line) shows, there are systematic deviations between fit and experimental data below 100 K, where the isomer shift is not constant as predicted by the model. This result points to a further coupling of the heme to additional low-frequency modes. We thus use a two-mode model to analyze the data following Eqs. 6–8:

$$\delta S = \delta S_{\text{chem}} - a \coth(h\nu_1/2\, k_B T) - b \coth(h\nu_2/2\, k_B T) \quad (9)$$

A two-mode model with frequencies at $\nu_1 = 30 \pm 20$ cm^{-1} and at $\nu_2 = 500 \pm 50$ cm^{-1} reproduces the data of myo-

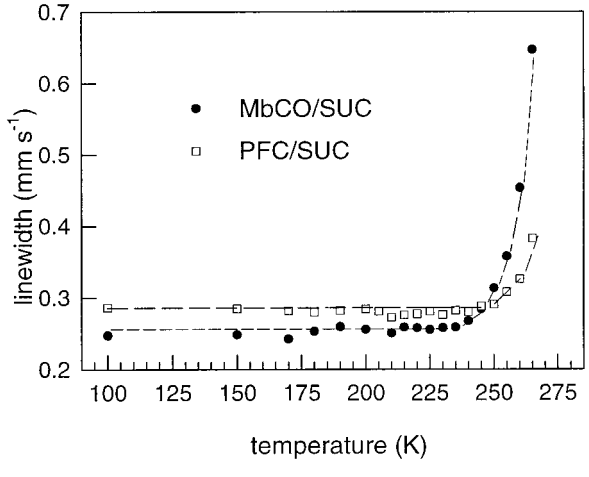

FIGURE 3 Experimental linewidths as a function of temperature: potassium-ferrocyanide and MbCO in 80% sucrose/water. The lines are drawn to guide the eye. The theoretical limit determined by the natural linewidth would be 0.2 mm/s.

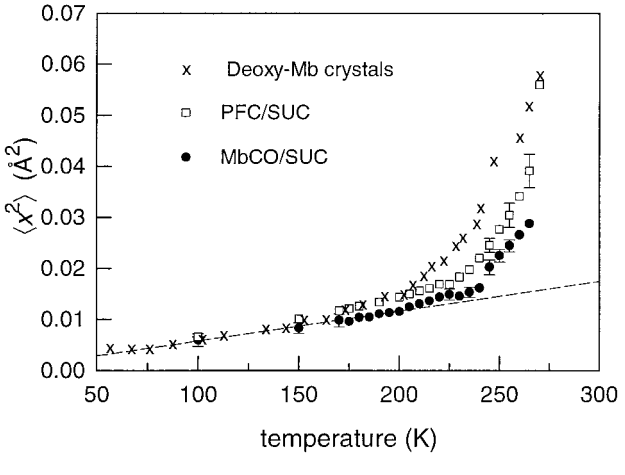

FIGURE 4 Mean square displacements, $\langle x^2 \rangle$, of ^{57}Fe versus temperature: potassium-ferrocyanide in 80% sucrose/water (□), MbCO in 80% sucrose/water (●), and deoxy-myoglobin crystals (×; Parak et al., 1982) for comparison. The dashed line represents a linear extrapolation of $\langle x^2 \rangle$ to $T = 0$ K.

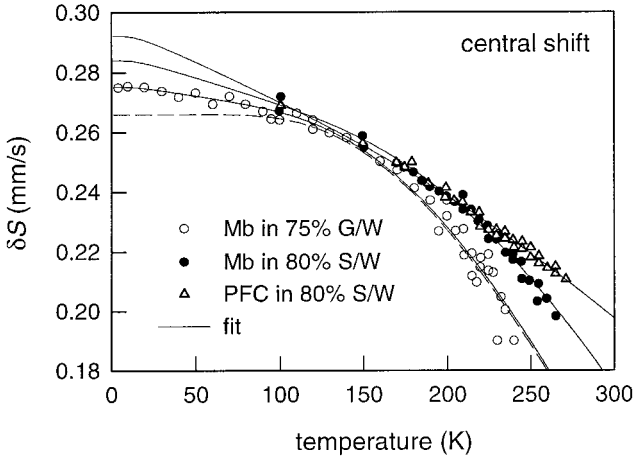

FIGURE 5 Central shift, δS, of the Mössbauer absorption lines as a function of temperature: Mb in 75% glycerol/water (○; Franke, 1992), Mb in 80% sucrose/water (●), and potassium-ferrocyanide (PFC) in 80% sucrose/water (△). The lines represent fits of the two-mode model, Eq. 9, to the data. The dashed line refers to a fit using a single effective mode. The parameters are given in Table 1.

globin in 75% glycerol/water within the entire temperature range as shown in Fig. 5. We next fit the MbCO/SUC data using the same set of parameters as starting values. This produces reasonable agreement between data and the model prediction. Moreover, the resulting parameters are the same within experimental error except for ν_2, which increases up to 630 ± 50 cm^{-1}. The same model can also account for the temperature dependence of the isomer shift of PFC, yielding $\nu_2 = 580 \pm 50$ cm^{-1}. However, in this case a single component fits the data equally well with the effective frequency of 250 ± 20 cm^{-1}. The respective parameters are given in Table 1.

Fig. 6 shows that the quadrupole splitting parameter increases steadily with the temperature up to 250 K where a strong enhancement is observed. The increase of this quantity could be interpreted as indicating a larger asymmetry of the heme environment.

DISCUSSION

A major goal of our investigation was to elucidate the dynamics of the heme-solvent coupling in the case of myoglobin and its potential relevance to function. The most pronounced changes depending on the temperature and the solvent concern the width of and the total area covered by the Mössbauer resonance line. Furthermore, it is striking that the heme iron buried in myoglobin (MbCO/SUC) and the solvent-exposed iron of PFC display similar behavior in the temperature dependence of their resonance lines. Quite analogous results were reported for iron salts dissolved in glycerol/water (Parak et al., 1989; Nienhaus et al., 1991; Franke, 1992). The respective onset temperature in this solvent was ~220 K. Similar to our results with PFC in sucrose/water, the extra loss of area in the Mössbauer probes in glycerol/water is accompanied by a broadening of the central Lorentzian line and additional broad wings in the spectrum that have to be accounted for by a second Lorentzian component. Alternatively a single non-Lorentzian component, a Cole-Davidson function, could fit the data just as well. Furthermore, the resulting average relaxation times were compatible to those derived for the viscoelastic (α) relaxation of the glycerol by other methods (Nienhaus et al., 1991). This result allows one to assign the line broadening to the structural relaxation of the solvent.

For myoglobin in 75% glycerol/water the onset of anharmonic mean square displacements occurs at ~215 K (Fig. 8 *a* and Franke, 1992). The broadening of the central line becomes prominent at slightly higher temperatures between 220 K and 230 K. The viscoelastic relaxation time of 75% glycerol/water in this temperature interval decreases from 2 μs to 200 ns (Kleinert et al., 1998), which is in the range of the ^{57}Fe-nuclear lifetime. This result, as in the case of the iron salt discussed above, is consistent with the notion that the broadening of the resonance line reflects the viscoelastic relaxation of the glycerol/water mixture. The coupling of the heme displacements to the solvent may involve the propionic acid side chains of the heme, which are exposed to the solvent. With myoglobin in the more viscous solvent, 80% sucrose/water, we observe the same spectral features except that the onset temperature is up-shifted to ~240 K. Significant line broadening is observed above 250 K (Fig. 3). For 80% sucrose/water at 250 K we derive a structural relaxation of 100 ms (Kleinert et al., 1998), which is several orders in magnitude larger than the nuclear lifetime. Thus, in contrast to glycerol/water, the structural relaxation of the bulk solvent does not contribute to line broadening. In the case of PFC in sucrose/water, however, the onset of line broadening occurs at slightly higher temperatures, 260–265 K (Fig. 3), where the bulk viscoelastic relaxation time has decreased to ~6 μs. In this range, line broadening should become noticeable. The difference in the onset temperatures therefore suggests that the microviscosity of the solvent in the vicinity of the protein is lower than in the bulk as probed by PFC. Such a reduction in viscosity due to preferential hydration of the protein surface at high co-solvent concentrations has been invoked to account for deviations from

TABLE 1 Central shift parameters

System	δS_{chem} (mm/s)	a (mm/s)	ν_1 (cm-1)	b (mm/s)	ν_2 (mm/s)
Mb 75% G/W	0.81 (±0.05)	0.002 (±0.0005)	30 (±20)	0.53 (±0.05)	500 (±50)
Mb 75% G/W	0.74			0.47	440
Mb 80% S/W	0.85	0.004	30	0.57	630
PFC 80% S/W	0.16	0.006	30	0.14	600

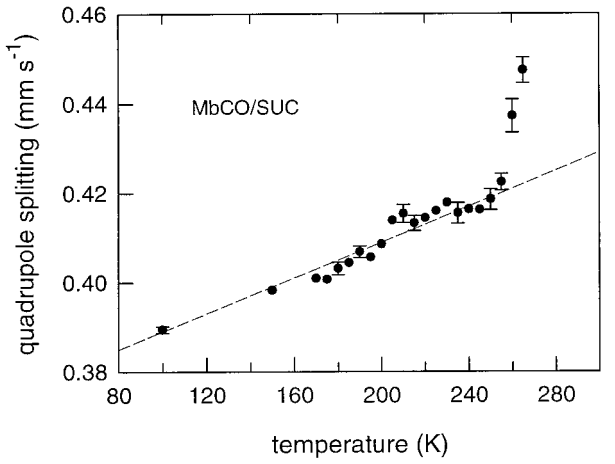

FIGURE 6 Quadrupole splitting of the Lorentzian doublets of MbCO in 80% sucrose/water versus temperature. The dashed line is shown to guide the eye.

Stokes law in the kinetics of ligand binding to myoglobin in this solvent (Kleinert et al., 1998).

The simultaneous broadening and loss of area of the resonance line point to an additional fast relaxation process resulting in a broad component whose width is larger than the accessible velocity window. To fit the data in the expanded velocity window we had to introduce a second Lorentzian doublet. In more general terms, this effect may be considered as the result of nonexponential relaxation together with the experimental limitation to discriminate small spectral contributions from the background. In the study of the iron salt in glycerol the spectra were analyzed using a Cole-Davidson function that exhibits high-frequency wings (Nienhaus et al., 1991). This model could account only in part for the loss in area. The missing area was assigned to a fast diffusive process. Fast restructuring of the protein-water hydrogen bond network on a picosecond time scale has been observed in neutron-scattering experiments of hydrated myoglobin (Doster et al., 1989; Settles and Doster, 1996). The anharmonic displacements of the nonexchangeable protein hydrogen atoms show the same onset temperature as the iron in myoglobin crystals, ~200 K, suggesting a common mechanism (Demmel et al., 1997).

The isomer shift δS provides additional information about the vibrational coupling of the heme to protein and solvent modes. As the second-order Doppler shift is biased toward high-frequency vibrations, which are much less anharmonic than modes in the range of a few wave numbers, it its plausible that a harmonic model works quite well in the entire range of temperatures. An effective two-mode model with frequencies centered at 30 cm^{-1} and 500–600 cm^{-1} fits the data reasonably well and is also consistent with known spectral features. Vibrations of surface side chains of proteins librate with frequencies near 30 cm^{-1}, which leads to a prominent band in the low-frequency neutron and Raman scattering spectra (Diehl et al., 1997). A line shape analysis of the Soret $\pi \rightarrow \pi^*$ transition of MbCO also suggest a coupling of the heme to low-frequency modes below 50 cm^{-1} (Ŝrajer et al., 1986). It was shown that the harmonic part of the iron mean square displacements can be explained according to Eq. 5 using a single dominant mode at 25 cm^{-1} (Wise et al., 1987). Furthermore, water exhibits a broad band centered at 50 cm^{-1} possibly due to intermolecular flexing modes that may interact with low-frequency protein vibrations. The situation is less transparent at high frequencies; resonance Raman experiments have identified the Fe-CO stretching vibration at 512 cm^{-1} (Tsubaki et al., 1982), which is close to the derived 500 cm^{-1} mode (Table 1). One should also expect the Fe-His at ~220 cm^{-1} to contribute to the second-order Doppler shift. However, adding this mode to the model did not improve the fits significantly. The most interesting aspect is the striking dependence of the high-frequency component on the solvent composition. The main difference in the fit parameters between 75% glycerol/water and the more viscous 80% sucrose/water is the upshift of ν_2 from 500 to 630 cm^{-1} (Table 1). The viscosity increase between 75% glycerol/water and 80% sucrose/water can be attributed, apart from a steric contribution, to stronger hydrogen bonds (Demmel et al., 1997). The increase in ν_2 thus indicates that the variation in the solvent force constants affects the heme libration. It is difficult to imagine that the Fe-CO stretching vibration should be so sensitive to the solvent composition. This would imply the presence of a bulky sucrose molecule inside the heme pocket. More likely appears a direct coupling between heme and solvent via the propionate side chains. In this frequency range water displays a broad band centered at 560 cm^{-1} due to hindered rotation which is strong in Raman and neutron scattering spectra (Sokolov et al., 1995; Settles et al., 1996).

Fig. 6 displays the temperature dependence of the quadrupole splitting parameter. In general, one expects the quadrupole splitting to decrease with the temperature as a result of motional averaging of the electrical field gradients. The opposite behavior is observed here. Two types of explanations, may be invoked: static and dynamic. A conformational drift with temperature affecting the local symmetry at the heme could play a role; the infrared spectrum of CO-ligated myoglobin shows three CO stretching bands corresponding to the conformational states A_0, A_1, and A_3 (Johnson et al., 1996) that differ in the CO-HisE7 interaction. The dominant conformation at physiological pH is A_1, but a conformational drift was observed above T_g of the solvent (75% glycerol/water), increasing the population of the tight state A_3. The increase in the quadrupole splitting of the MbCO-Mössbauer absorption lines may thus indicate a larger field gradient in state A_3. Wise et al. (1987) have proposed a dynamical origin; the effect of vibronic coupling on the electric field gradient and the motion of the ligand can lead to a temperature-dependent quadrupole splitting. The drastic increase in the splitting parameter at a well defined onset temperature argues in favor of a dynamical origin.

The above results suggest a tight coupling of heme displacements, structural relaxation of the solvent, and solvent vibrational modes. How do these correlations affect the kinetics of ligand binding to myoglobin? In flash photolysis experiments on CO-myoglobin, one observes two kinetic components, a fast intramolecular recombination process and bimolecular binding from the solvent. The simplest model involves two kinetic barriers, an outer barrier, controlling the ligand transfer from the solvent to the heme pocket, and an inner barrier, related to bond formation with the heme iron. In a recent flash photolysis study of MbCO we have investigated the solvent dependence of these barriers (Kleinert et al., 1998). The intramolecular barrier was found to be solvent independent. The outer barrier, however, was changing with the solvent viscosity consistent with Kramers law of activated escape at high friction. Fig. 7 compares kinetic data to the Mössbauer results: a) the mean square displacements of the heme iron in two solvents and b) the escape fraction of the ligand after photolysis. The ligand escape fraction to the solvent N_S is determined by a competition between the outer and the inner barrier. At ambient temperatures and at low viscosity the inner barrier dominates, which leads to $N_S \approx 1$. The opposite is true at high viscosity or low temperatures, where the outer barrier dominates the temperature dependence of N_S. The figure illustrates that the heme anharmonic displacements and the ligand displacements out of the protein display identical temperature shifts in response to modification of the co-solvent. This suggests that both types of motions are connected to the same solvent structural fluctuations. The two solvents differ mainly in their viscosity at a given temperature. If the bulk viscosity of the solvent is the essential control variable, one should expect identical viscosities at the respective onset temperatures. The viscosities that are indicated in Fig. 7 *a* differ, however, by several orders in magnitude. This result suggests again that myoglobin in 80% sucrose/water is preferentially hydrated to a larger extent than in 75% glycerol/water. The two vertical lines in Fig. 7 *b* denote the position of the glass temperatures of the two solvents. The onset temperature in 75% glycerol/water is located 40° above the glass temperature T_g, whereas in 80% sucrose/water onset temperature and glass temperature nearly coincide. A discrepancy of 40° is reasonable according to the estimates of the bulk viscosity and the corresponding structural relaxation times given above. The coincidence of the two temperatures would imply a relevant time scale of 100 s, the characteristic time of structural relaxation at T_g. This process is far too slow to account for the observed ligand escape fraction and the onset of anharmonic displacements seen by Mössbauer spectroscopy in sucrose/water. The discrepancy can be resolved if the microviscosity at protein-solvent interface is much lower than in the bulk. Partial demixing of water and co-solvent in the protein solvation shell would drastically reduce the relevant relaxation times. Thermodynamic experiments determine a lower preferential hydration for glycerol than for sucrose (Timasheff, 1993, 1995; Lin and Timasheff, 1996). This result together with the lower co-solvent concentration may explain why the microviscosity for 75% glycerol/water in the solvation shell is close to the bulk value. For 80% sucrose/water our dynamic approach detects an excess number of water molecules in the solvation shell of myoglobin as suggested by the thermodynamic analysis. This result supports the view that the stabilizing action of sucrose is achieved by preferential exclusion from the protein domain. This does not, however, exclude the direct interaction of sucrose molecules with the protein surface, which was proposed by Carpenter and Crowe (1988a). On the contrary, the viscosity of the sucrose/water solvation shell is much larger than in water, which requires the presence of sucrose molecules. At the transition temperature of 230 K, we expect it to be close to the viscosity of glycerol/water at 210 K, which yields 10^4 Poise (Fig. 7 *a*). Increasing the temperature to 293 K lowers the viscosity of the sucrose/water solvation shell to ~2 Poise. This value has to be compared with the bulk viscosity, which is 200 Poise at room temperature (Kleinert et al., 1998).

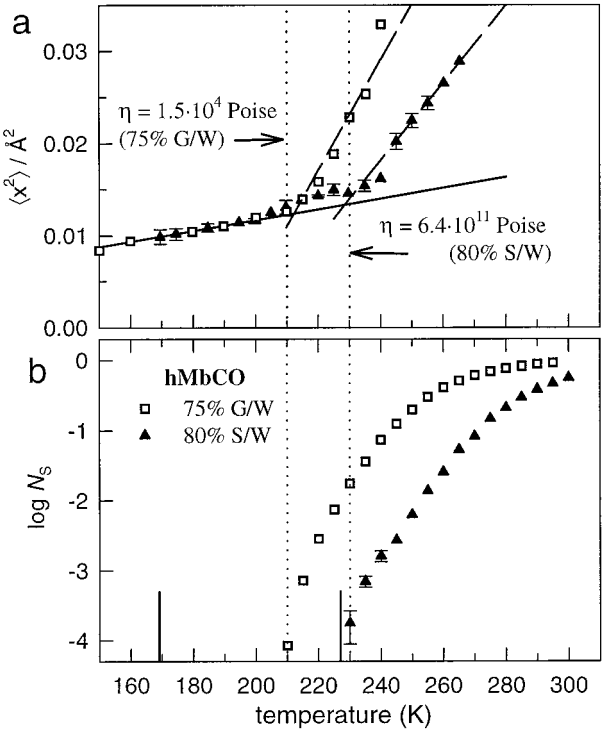

FIGURE 7 (*a*) Mean square displacements of the heme iron of myoglobin in 75% glycerol-water (□; Franke, 1992) and 80% sucrose/water (▲). The bulk solvent viscosity at respective onset temperature is also given. The lines are drawn to guide the eye. (*b*) Escape fraction N_s of MbCO in 80% sucrose/water (▲, 80% S/W), 75% glycerol-water (□, 75% G/W) versus temperature. The bars indicate the calorimetric glass temperatures T_g of 80% sucrose/water and 75% glycerol-water at ~230 K and ~170 K, respectively.

The kinetics of intramolecular bond formation of CO-myoglobin involves a translation of the iron relative to the heme plane. The observed solvent independence of this process (Kleinert et al., 1998) shows that the main iron

displacements do not couple directly to the reactive coordinate. This result is corroborated by molecular dynamics simulations of myoglobin (Kuczera et al., 1990) where it was found that the out-of-plane motions contribute less than 10% to the total iron displacements. The dominant contribution comes from a shift parallel to the heme plane that the authors attribute to a sliding motion of the heme as a whole in the protein cleft. Analogous results were found in a normal mode analysis of myoglobin (Melchers et al., 1996).

One of the unsolved puzzles in this field is related to the observation that the kinetics of intramolecular ligand binding is always polychromatic (Austin et al., 1975). A distribution of activation enthalpies, reflecting the conformational heterogeneity of the protein structure, was invoked as a plausible explanation (Frauenfelder et al., 1988, 1991), but the molecular origin of the distribution is still obscure. We now discuss whether the local diffusion of the heme in its protein cleft may contribute to the observed nonexponential kinetic shape of intramolecular ligand binding. Assume that the position and orientation of the heme that is sliding modulates the inner barrier. The sliding motion of the heme distorts the geometry on the proximal site of the heme via the covalent attachment of the iron to the imidazole side chain of HisF8. This correlation is likely to induce a cross-coupling of sliding and out-of-plane modes of the iron-porphyrin. The respective fluctuations may be small but still large enough to modulate the inner barrier by several kJ/mol.

If the crossing of the inner barrier by the ligand occurs on a time scale that is faster than the viscoelastic sliding, an apparent static distribution of barrier heights will result. The above analysis indicates that the structural relaxation of the solvent is the main factor that determines the rate of heme sliding. It follows that the observed barrier distribution should change significantly when the rate of viscoelastic relaxation starts to exceed the intramolecular binding rate. In the case of CO-horse myoglobin in 75% glycerol/water, the crossover takes place between 210 K and 220 K on a time scale of $\sim$1–2 μs (Post et al., 1993; Kleinert et al., 1998). It is important to stress that a single temperature-independent enthalpy spectrum is able to reproduce the polychromatic kinetics of the final binding step, B$\rightarrow$A, in the temperature range between 60 K and 200 K. Above 210 K, the kinetic curves change very little with temperature in contrast to what is derived from the enthalpy spectrum (Figs. 7 *a* and 9 in Post et al., 1993). The activation enthalpy distribution apparently becomes temperature dependent when ligand rebinding and heme sliding start to overlap on the same time scale.

In a theoretical analysis, Sastry and Agmon (1997) introduce a conformational change to explain this feature, which they call collapse. The transition supposedly reduces the difference between the equilibrium conformations of bound and deoxy hemes. Myoglobin embedded in a trehalose glass does not show this transition, suggesting structural arrest due to the large solvent viscosity (Agmon and Sastry, 1997). The model of an effective temperature-dependent potential fits the data quite well. It does not, however, explain why the solvent-coupled structural change takes place 40° above the glass temperature.

CONCLUSION

As an important result of this study we consider the observation that the solvent-exposed iron of potassium ferrocyanide (PFC) and the heme iron buried in the globin cleft of myoglobin display a remarkably similar temperature dependence in their Mössbauer spectra. As the PFC dynamics reflects essentially the viscoelastic relaxation of the solvent, it is difficult to avoid the analogous conclusion in the case of the heme protein. It has been previously recognized that the displacements of the heme iron of myoglobin crystals, and of the nonexchangeable hydrogens of D_2O-hydrated myoglobin, measured by neutron scattering, display a parallel temperature dependence (Doster et al., 1989). The dominant fluctuational amplitudes of the latter were assigned to motions of polar side chains on the protein surface (Diehl et al., 1997). The parallel temperature dependence then suggests that the heme motions resemble those of a polar side chain. The role of the heme as a monitor of intramolecular protein dynamics may have been overemphasized in the past. The slight anharmonic increase in the displacements observed with Mössbauer spectroscopy (Parak et al., 1987) on dehydrated myoglobin gives an idea of the protein-intrinsic contribution to the iron displacements, which is in the range of 10–20% of the total amplitude.

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REFERENCES

Ansari, A., C. M. Jones, E. R. Henry, J. Hofrichter, and W. A. Eaton. 1992. The role of solvent viscosity in the dynamics of protein conformational changes. *Science.* 256:1796–1798.

Ansari, A., C. M. Jones, E. R. Henry, J. Hofrichter, and W. A. Eaton. 1994. Conformational relaxation and ligand binding in myoglobin. *Biochemistry.* 33:5128–5145.

Austin, R. H., K. W. Beeson, L. Eisenstein, H. Frauenfelder, and I. C. Gunsalus 1975. Dynamics of ligand binding to myoglobin. *Biochemistry.* 14:5355–5373.

Beece, D., L. Eisenstein, H. Frauenfelder, D. Good, M. C. Marden, L. Reinisch, A. H. Reynolds, L. B. Sorensen, and K. T. Yue. 1980. Solvent viscosity and protein dynamics. *Biochemistry.* 19:5147–5157.

Carpenter, J. F., and J. H. Crowe. 1988a. The mechanism of cryoprotection of proteins by solutes. *Cryobiology.* 25:244–255.

Carpenter, J. F., and J. H. Crowe. 1988b. Modes of stabilization of a protein by organic solutes. *Cryobiology*. 25:459–470.

Carpenter, J. F., and J. H. Crowe. 1989. An infrared spectroscopic study of the interactions of carbohydrates with dried proteins. *Biochemistry.* 28:3916–3922.

Crowe, L. M., D. S. Reid, and J. H. Crowe. 1996. Is trehalose special for preserving dry biomaterials? *Biophys. J.* 71:2087–2093.

Demmel, F., W. Doster, W. Petry, and A. Schulte. Vibrational frequency shifts as a probe of hydrogen bonds: thermal expansion and glass transition of myoglobin in mixed solvents. 1997. *Eur. Biophys. J.* 26:327–335.

Diehl, M., W. Doster, W. Petry, and H. Schober. 1997. Water-coupled low frequency modes of myoglobin and lysozyme observed by inelastic neutron scattering. *Biophys. J.* 73:2726–2732.

Doster, W., S. Cusack, and W. Petry. 1989. Dynamic transition of myoglobin revealed by inelastic neutron scattering. *Nature.* 337:754–756.

Doster, W., S. Cusack, M. Settles, and F. Post. 1993. Hydration effects on protein function: low frequency modes of partially hydrated proteins. *Proc. Water-Biomolecule Interactions Conf. Palermo*. 43:123–126.

Franke, M. 1992. Konformationssubzustände in Myoglobin, Mössbauerspektroskopische Untersuchung und modellmässige Deutung der Reaktionskinetik mit CO. Ph.D. thesis. Friedrich-Alexander-Universität, Erlangen-Nürnberg, Germany.

Frauenfelder, H., F. Parak, and R. D. Young. 1988. Conformational substates in proteins. *Annu. Rev. Biophys. Biophys. Chem.* 17:451–479.

Frauenfelder, H., S. G. Sligar, and P. G. Wolynes. 1991. The energy landscapes and motions of proteins. *Science*. 254:1598–1603.

Gottfried, D. S., E. S. Peterson, A. G. Sheikh, J. Wang, M. Yang, and J. M. Friedman. 1996. Evidence for damped hemoglobin dynamics in a room temperature trehalose glass. *J. Phys. Chem.* 100:12034–12042.

Greenwood, N. N., and T. C. Gibb. 1972. Mössbauer Spectroscopy. Chapman and Hall, London.

Hagen, S. J., J. Hofrichter, and W. A. Eaton. 1995. Protein reaction kinetics in a room temperature glass. *Science.* 269:959–962.

Hagen, S. J., J. Hofrichter, and W. A. Eaton. 1996. Geminate rebinding and conformational dynamics of myoglobin embedded in a glass at room temperature. *J. Phys. Chem.* 100:12008–12021.

Housley, R. M., and F. Hess. 1966. Analysis of Debye-Waller and Mössbauer-thermal-shift measurements. I. General theory. *Phys. Rev.* 146: 517–526.

Johnson, J. B., D. C. Lamb, H. Frauenfelder, J. D. Müller, B. MacMahon, G. U. Nienhaus, and R. D. Young. 1996. Ligand binding to heme proteins. VI. Interconversion of taxonomic substates in carbonmonoxymyoglobin. *Biophys. J.* 71:1563–1573.

Keller, H., and P. G. Debrunner. 1980. Evidence for conformational mean square displacements in frozen aqueous solution of oxymyoglobin. *Phys. Rev. Lett.* 45:68–71.

Kleinert, T., W. Doster, H. Leyser, W. Petry, V. Schwarz, and M. Settles. 1998. Solvent composition and viscosity effects on the kinetics of CO-binding to horse myoglobin. *Biochemistry*. 37:717–733.

Knapp, E. W., S. F. Fischer, and F. Parak. 1982. Protein dynamics from Mössbauer spectra: the temperature dependence. *J. Phys. Chem.* 86: 5042–5047.

Kuczera, K., J. Kuriyan, and M. Karplus. 1990. Temperature dependence of the structure and dynamics of myoglobin: a simulation approach. *J. Mol. Biol.* 213:351–373.

Lin, T. Y., and S. N. Timasheff. 1996. On the role of surface tension in the stabilization of globular proteins. *Protein Sci.* 5:372–381.

Melchers, B., E. W. Knapp, F. Parak, L. Cordone, A. Cupane, and M. Leone. 1996. Structural fluctuations of myoglobin from normal mode, Mössbauer, Raman, and absorption spectroscopy. *Biophys. J.* 70: 2092–2099.

Nadler, W., and K. Schulten. 1984. Theory of Mössbauer spectra of proteins fluctuating between conformational substates. *Proc. Natl. Acad. Sci. USA.* 81:5719–5723.

Nienhaus, G. U., H. Frauenfelder, and F. Parak. 1991. Structural fluctuations in glass forming liquids: Mössbauer spectroscopy on iron in glycerol. *Phys. Rev. B.* 43:3345–3349.

Nowik, I., S. G. Cohen, E. R. Bauminger, and S. Ofer. 1983. Mössbauer absorption in overdamped harmonically bound particles in Brownian motion. *Phys. Rev. Lett.* 50:1528–1531.

Parak, F., M. Fischer, E. Graffweg, and H. Formanek. 1987. Distributions and fluctuations of protein structures investigated by x-ray analysis and Mössbauer spectroscopy. *In* Structure and Dynamics of Nucleic Acids, Proteins, and Membranes. E. Clementi, and S. Chin, editors. Plenum Press, New York. 139–148.

Parak, F., M. Fischer, and G. U. Nienhaus. 1989. The similarity in the dynamics of myoglobin and glycerol as seen from Mössbauer spectroscopy on ^{57}Fe. *J. Mol. Liquids.* 42:145–153.

Parak, F., and H. Formanek. 1971. Untersuchung des Schwingungsanteils und des Kristallgitterfehleranteils des Temperaturfaktors in Myoglobin durch Vergleich von Mössbauerabsorptionsmessungen mit Röntgenstrukturdaten. *Acta Crystallogr. A*. 27:573–578.

Parak, F., E. N. Frolov, R. L. Mössbauer, and V. I. Goldanskii. 1981. Dynamics of metmyoglobin crystals investigated by nuclear gamma resonance. *J. Mol. Biol.* 145:825–833.

Parak, F., E. W. Knapp, and D. Kucheida. 1982. Protein dynamics: Mössbauer spectroscopy on deoxy-myoglobin crystals. *J. Mol. Biol*. 161: 177–194.

Perutz, M. F. 1989. Myoglobin and hemoglobin, role of distal residues in reactions with heme ligands. *Trends Biochem. Sci*. 14:42–44.

Post, F., W. Doster, G. Karvounis, and M. Settles. 1993. Structural relaxation and nonexponential kinetics of CO-binding to horse myoglobin. *Biophys. J.* 64:1833–1842.

Reinisch L., J. Heidemeier, and F. Parak. 1985. Determination of the second order Doppler shift of iron in myoglobin by Mössbauer spectroscopy. *Eur. Biophys. J.* 12:167–172.

Ringe D., G. Petsko, D. Kerr, and P. R. O. de Montellano. 1984. Reaction of myoglobin with phenylhydrazine: a molecular doorstop. *Biochemistry*. 23:2–4.

Sastry, G. M., and N. Agmon. 1997. Trehalose prevents myoglobin collapse and preserves its internal mobility. *Biochemistry*. 36:7097–7108.

Settles, M., and W. Doster. 1996. Anomalous diffusion of protein hydration water, a neutron scattering study of hydrated myoglobin. *Faraday Discussion*. 103:169–279.

Sokolov A. D., J. Hurst, and D. Quitmann 1995. Dynamics of supercooled water. *Phys. Rev. B.* 51:12865–12868.

Ŝrajer, V., K. T. Schomacker, and P. M. Champion. 1986. Spectral broadening in biomolecules. *Phys. Rev. Lett*. 57:1267–1270.

Timasheff, S. N. 1993. The control of protein stability and association by weak interactions with water. *Annu. Rev. Biophys. Biomol. Struct.* 22: 67–97.

Timasheff, S. N. 1995. Preferential interactions of waters and cosolvents with proteins. *In* Protein-Solvent Interactions. R. B. Gregory, editor. Marcel Dekker, New York. 445–482.

Trautwein A., H. Eicher, and A. Mayer. 1970. Electronic structure, quadrupole splitting, chemical shift and susceptibility of ferrous iron in anhydrohemoglobin, anhydromyoglobin, and bispyridinehemin. *J. Chem. Phys*. 52:2473–2477.

Tsubaki M., R. B. Srivastava, and N. T. Yu. 1982. Resonance Raman investigation of carbon monoxide bonding in hemoglobin and myoglobin. *Biochemistry.* 21:1132–1140.

Wise, W. W., G. C. Wagner, and P. G. Debrunner. 1987. Dynamic features of the Mössbauer spectra of heme proteins. *In* Protein Structure, Molecular and Electronic Reactivity. R. Austin, E. Buhks, B. Chance, D. De Vault, P. L. Dutton, H. Frauenfelder, and V. I. Gol'danskii, editors. Springer Verlag, Berlin. 509–510.

Yancey, P. H., M. E. Clark, S. Hand, R. Bowlus, and G. N. Somero. 1982. Living with water stress: evolution of osmolyte systems. *Science.* 217: 1214–1222.